IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Ebrahim ZANDI, et al.

Title: COMPOSITION AND METHOD
           FOR RECONSTITUTING IkB
           KINASE IN YEAST AND
           METHODS OF USING SAME

Appl. No.:   10/079,949

Filing Date: 2/19/2002

Examiner: Prouty, Rebecca E.

Art Unit: 1652

Confirmation   6542
Number:

SUPPLEMENTAL DECLARATION UNDER 37 CFR SECTION 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1.   We, Ebrahim Zandi and Beth Schomer Miller, hereby declare as follows.

2.   We are the Ebrahim Zandi and Beth Schomer Miller, who are named as co-inventors of the above-identified application.

3.   That we conceived and reduced to practice in the United States the transformation of an IKK subunit gamma (γ) gene, an IKK subunit alpha (α) gene and/or an IKK subunit beta (β) gene into yeast and the separation from that yeast a substantially homogenous and biologically functional IKK protein complex prior to November 15, 2000, the online publication date of the literature article Li et al. (2001) "Role of IKKγ/NEMO in Assembly of the IkB Kinase Complex"

Journal of Biological Chemistry 276(6):4494-4500. Attached hereto is Exhibit A, a copy of pages from laboratory notebooks recorded by Beth Schomer Miller working under our direct control and supervision showing a reduction to practice wherein the activity of a purified IKK complex from yeast transformed with either IKKβ, IKKβγ, or IKKαβγ compared to mammalian IKK complex isolated from control Hela cells or TNF stimulated HELA cells was determined. These experimental results demonstrate that a yeast cell was transformed with an IKK subunit gamma (γ) gene, an IKK subunit alpha (α) gene and/or an IKK subunit beta (β) gene. The yeast was then grown and a substantially homogenous and biologically functional IKK protein complex was separated from the yeast.

4.   That the documents in Exhibit A, which relates to the aforementioned actual reduction to practice, are exact and true copies. All personal information, including names and dates have been redacted from the documents, but all dates are prior to November 15, 2000.

5.   Attached hereto is Exhibit B. Exhibit B is a typed version of Exhibit A and a true and exact representation of the handwritten information of Exhibit A.

6.   As specifically identified on pages 1 and 9 of Exhibit B, the purpose of the experiment was to compare the activity of recombinantly produced IKK complexes that were isolated from yeast transformed with IKK subunits alpha, beta, and gamma (yαβγ), subunits beta and gamma (yβγ), subunit beta (yβ), to IKK complexes isolated from non-stimulated Hela cells (HNS) and TNF-stimulated Hela cells (TNF). The remaining pages set forth the experimental protocols, the resulting activity of the isolated IKK complexes and the amount of IKKβ subunit present in the isolated IKK complexes. The gels shown on pages 4, 5, 10 and 11 of Exhibit B demonstrate that the activity of recombinantly produced IKK complex isolated from yeast transformed with IKK subunits alpha, beta, and gamma (yαβγ) is higher than purified IKK complex from non-stimulated HeLa cells and the same or slightly higher than purified activated IKK complex from TNF-stimulated Hela cells. Similar gels and results are show in Figure 3 and on page 15, lines 21 to 27 of the above-identified application.

Atty. Dkt. No. 064189-0501

7.   We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are true; and further that all statements made herein are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United Sates Code, and that such false statements may jeopardize the validity of legal decisions of any nature based on them.

Respectfully submitted,

Ebrahim Zandi

Signature: _Ebra him Zandi._   Date: _02 – 10 – 2009_

Beth Schomer Miller

Signature: _____   Date: _____

Atty. Dkt. No. 064189-0501

7.   We further declare that all statements made herein of our own knowledge are true and
that all statements made on information and belief are true; and further that all statements made
herein are made with the knowledge that willful false statements and the like so made are
punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United Sates
Code, and that such false statements may jeopardize the validity of legal decisions of any nature
based on them.

Respectfully submitted,

Ebrahim Zandi

Signature: _____   Date: _____

Beth Schomer Miller

Signature: _Bth Schau Millu_   Date: _10 Feb 09_

Exhibit A

Purpose: to compare IKK activity in
γαβδ   γαδ   γβ   HNS   TNF

Both βδ   8.25 good (HA) signal in 2 sec exp.
GF   5μL   Fr 10 or 11

Both αβδ   good α→δ 1min 5μL Fr 10-11
GF   1Δ γ←αβδ
              HA good signal 15 sec similar to

1Δ of ▮▮▮ γ→βδ Fr 10

Both β HA detect some Fr 15 after 1min (15Δ)

HNS GF ▮▮▮
     HNS β detected in 10 + 11 after 1min   20Δ
     TNF weakly detected in 10 + 11 40min   20Δ
          INP. identical.

Hugo's westerns were also poor for detection of
       β in Balance + αβδ in his assays.
       I'll have to play around with amounts

HNS Q20 + TNF Q20 were separated by gel filtration
INP. could detect S-15Δ by αIKK β + γ western
                                              in 15 sec.

       Less present than 5Δ γ IKK

Put fractions in gel filtration → ~10 fold dilution
would need to use 150Δ for same amt.
                        150(10) + 150(11)
Concentrate 300Δ → 30Δ

                use: 5.10.15

B-HA fraction 15 G.F.
3⅃ + 12⅃ 1x KA
5⅃ + 10⅃ 1x
10⅃ + 5⅃ 1x

Bγ -HA Fr 10
3⅃ + 12⅃ 1x
5⅃ + 10⅃ 1x
10⅃ + 5⅃ 1x

αBγ Fr 10-11
3⅃ + 12⅃ 1x
5⅃ + 10⅃ 1x

HNS Q 30 → Sp 6 GF 10+11
200 + 200 → 40⅃
use 5, 10, 15⅃
10⅃ 5⅃ 1x

TNF Q 30 → Sp 6 GF 10+11
200⅃ + 200⅃ → 40⅃
use 5, 10, 15⅃
10⅃ 5⅃ 1x

Load 35⅃ each

1	empty	✓
2	empty	✓
3	B3	✓
4	5	✓
5	10	✓
6	Bγ 3	✓
7	5	✓
8	10	✓
9	αBγ 3	✓
10	5	✓
11	HNS 5	✓
12	10	✓
13	5	✓
14	TNF 5	✓
15	10	✓
16	5	✓
17	mw	

all currently frozen

ULTAFREE MC
30, KD
put
300⅃ 1x kinase
buffer in
bottom
↓
prevent drying
filter

Should recover
≤ 40⅃ reinstate
recover
25⅃ HNS
add 15⅃ 1x
40⅃ TNF

1. aliquot extract + buffer according to table
2. Add 30⅃ kinase cocktail Inc 30' 30°C
3. add 6⅃ 6x SDS PAGE, heat
4. Load 10⅃ gel   Only 6x cock!

Cocktail - 15
10x kinase   45   ✓
20mm DTT   45   ✓
200mm ATP   45   ✓
0.5ng/ul GST-Iku 30⅃ ✓
γ ATP   7.5⅃   906.58
H2O   277.5 ✓

3⅃ 906÷+
445⅃ 58

Purpose: to compare activity of
yβ vs y$\beta\gamma$ vs y$\alpha\beta\gamma$ vs HNS vs TNF

10% gel (10·10)

30% acryl	5 μ	Stack
8.8	3.75	1.05
H$_2$O	6.25	1.9 (6.8)
APS	200 100	4.5
TEMED	20 10	75
		10

File/Range: D:\Users\1012bsm.gel / 0.000-45853 Counts / 1.000000
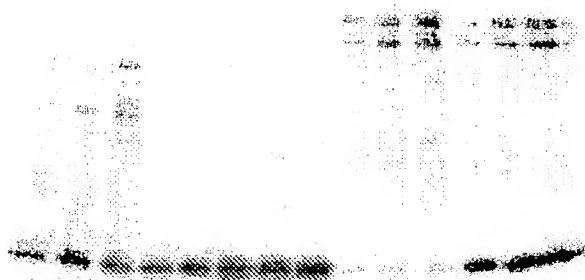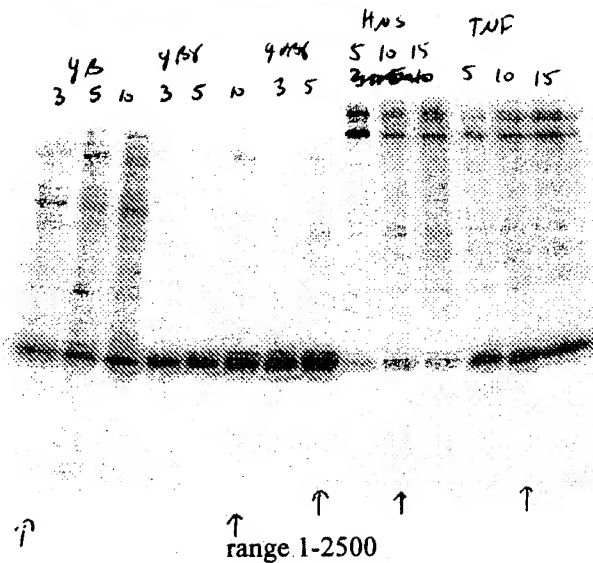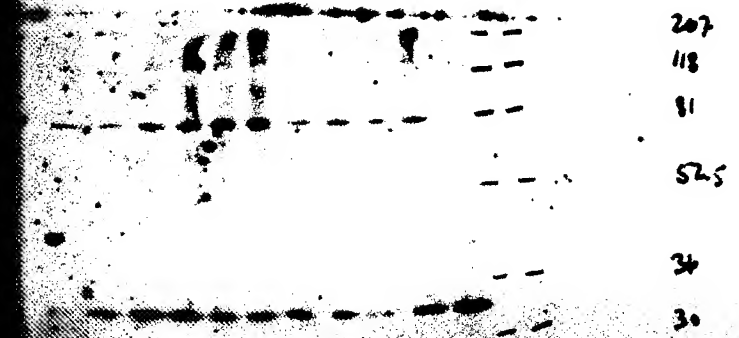User Name: phospho
Image Name: D:\Users\1012bsm.gel
Image Comment: yeast b bg abg HNS TNF-Hela
scanned 9:13 am to2:05 pm

Present Date/Time:
Scan Date/Time:
Prep. Date/Time:

range 1-10,000

File/Range: D:\Users\1012bsm.gel / 0.000-45853 Counts / 1.000000
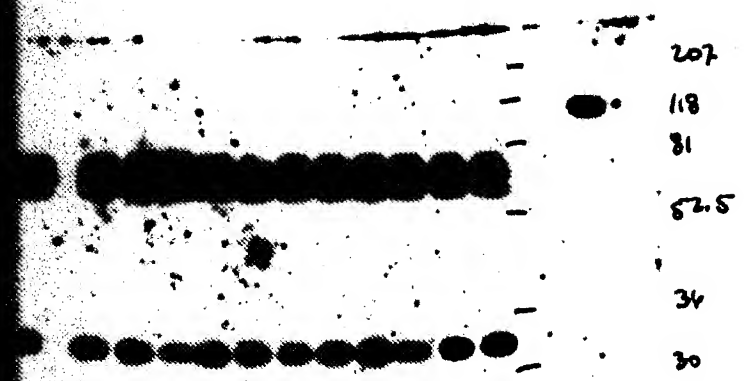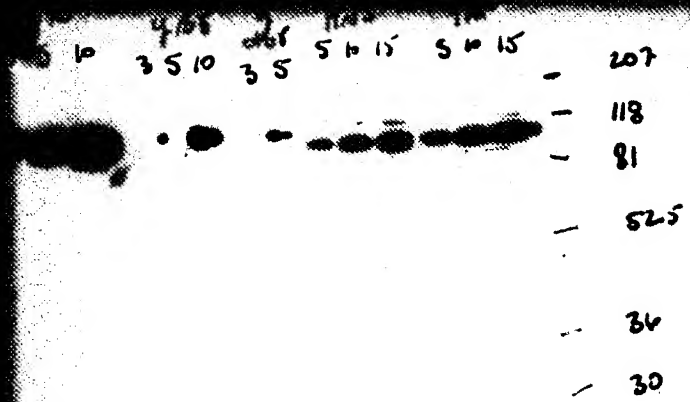User Name: phospho
Image Name: D:\Users\1012bsm.gel
Image Comment: yeast b bg abg HNS TNF-Hela
                        scanned 9:13 am to2:05 pm

Present Date/Time:
Scan Date/Time:
Prep. Date/Time:

range 1-2500

5 10   3 5 10   3 5   5 10 15   5 10 15 — 207

— 118
— 81

— 52.5

W: αIKKβ
1:500

— 36

— 30

Purpose: to compare IKK activity in
yb vs y/Br vs yaBr vs HNS vs TNF-Hela

repeat of 10-11 with attempt to use more similar amounts

HNS & TNF (Q20 → sup 6 GF 10+11)
  Put 300μ 1x IKinase buffer in bottom to prevent drying.
  Top: 200μ sup 6 GF 10 + 200μ sup 6 GF 11

  recover ~40μ + adjust vol. to 40μ
                              (1x IKA)

β-HA fraction 15   5μ + 45μ IKA
                    Dilute 1:10 in 1x kinase

Tube/lane				
2	0.5μ	5μ	+ 16μ	1x
3	1μ	10μ	+ 11μ	1x
4	2μ	20μ	+ 7μ	1x
5 Bγ	7μ	(~5μ Hela TNF)	+14μ	1x
6	14μ	~10	+7μ	1x
7	21μ	~15	+0μ	
8 aBγ	7		+14μ	1x
9	14		+7μ	1x
10	21		+0μ	
11 HNS	5		+16μ	1x
12	10		+11μ	1x
13	15		+6μ	1x
14 TNF	5		+16μ	1x
15	10		+11μ	1x
16	15		+6μ	1x
17	mw			

all loaded
correctly / 40μ
each.

21
35
—
56

1. Aliquot extract +
                    buffer

2. Add   35μ Kinase
   Cocktail   Inc 30' 30°C
              19μ 112

3. Add   HNS 6x
   SDS PAGE
   Heat 95 C 5'

4. Load 10% gel
              (40μ)

Cocktail	16 samples	+ 4
10x Kinase	48μ	12μ
20mm DTT	48μ	12μ
200mm ATP	48μ	12μ
GST-IkB	32μ	8μ
32P ATP	8μ	2μ
H2O	296	74
	480	120

906-58

10x DTT
20mm DTT
.02 1M + .98 H2O

File/Range: D:\Users\1017bsm.gel / 0.000-45853 Counts / 0.814331
User Name: phospho
Image Name: D:\Users\1017bsm.gel
Image Comment: 2 experiments
1. 3 M urea GF column fractions (concentrated)
2. yeast b, bg, abg, HNS, TNF stim Hela

Present Date/Time:
Scan Date/Time:
Prep. Date/Time:

scale 1-2500

File/Range: D:\Users\1017bsm.gel / 0.000-45853 Counts / 0.814331
User Name: phospho
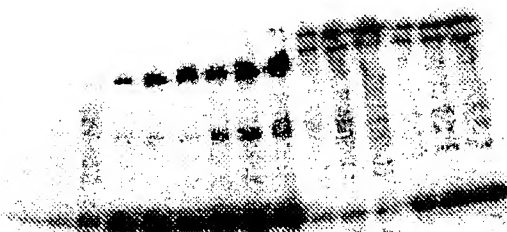Image Name: D:\Users\1017bsm.gel
Image Comment: 2 experiments
    1. 3 M urea GF column fractions (concentrated)
    2. yeast b, bg, abg, HNS, TNF stim Hela

Present Date/Time:
Scan Date/Time:
Prep. Date/Time:

scale 1-250

Exhibit B

Purpose: to compare IKK activity in $\gamma\alpha\beta\gamma$ $\gamma\beta\gamma$ $\gamma\beta$ HNS TNF

Beth $\beta\gamma$ 8-25 good HA signal in 2 sec exp.
GF   5μL   Fr 10 or 11

Beth $\alpha\beta\gamma$ good $\alpha+\gamma$ 1 min 5μL Fr 10-11
GF   1λ $\gamma\alpha\beta\gamma$
                         HA good signal 15 sec similar to
      1λ of _____ $\gamma \rightarrow \beta\gamma$ Fr 10

Beth βHA detect some Fr 15 after 1 min (15λ)

HNS GF
      HNS β detected in 10 + 11 aft 1 min   20λ
      TNF weakly detected in 10 + 11 40 min   20λ

      INP identical

      Hugo's westerns were also poor for detection of
         β in β alone + $\alpha\beta\gamma$ in his assays.
      I'll have to play around with amounts

HNS Q20 + TNF Q20 were separated by gel filtration
Inp. could detect 5 – 15λ by αIKK $\beta + \gamma$ Western in 15 sec.

      Less present than 5λ γIKK

Put fractions in gel filtration $\rightarrow$ ~ 10 fold dilution
      would need to use 150λ for same amt.

Concentrate 150(10) + 150(11) λ $\rightarrow$ 30λ

      use: 5 . 10 . 15

β-HA fraction 15 G.F.
    3λ + 12λ 1x KA
    5λ + 10λ 1x
    10λ + 5λ 1x

βγ-HA Fr 10
    3 + 12λ 1x
    5 + 10λ 1x
    10 + 5λ 1x

αβγ   Fr 10-11
    3 + 12λ 1x
    5 + 10λ 1x

HNS Q 20→ sup 6 GF 10 + 11
            200 + 200 →40λ
        use 5,   10,   15λ
            +   +
            10λ 5λ 1x

TNF Q 20 → sup 6 GF 10 + 11
            200 + 200 →40λ
        use 5,   10,   15λ
            +   +
            10λ 5λ
            1x   1x

1. Aliquot extract + buffer according to table
2. Add 30λ Kinase cocktail Inc 30' 30°C
3. Add 9λ 6x SDS PAGE, heat
4. Load 10% gel
                    — Only 6λ added

    Cocktail – 15
        10x Kinase   45
        20mm DTT   45
        200µm ATP   45
    0.5 mg/µl Gst - IκBα 30λ
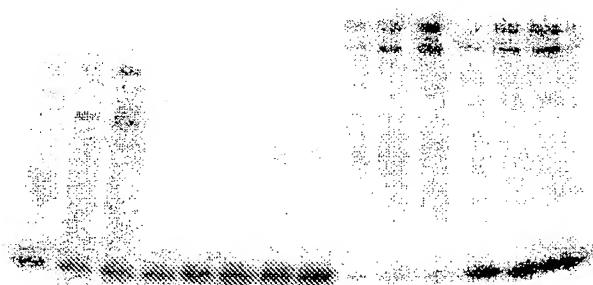   γ ATP   7.5λ 906-58   3λ 906-57
        H_2O 277.5   4.5λ 58

Load 35λ each
1   empty
2.   empty
3.   β 3
4.   5
5.   10
6.   βγ 3
7.   5   All correctly loaded
8.   10
9.   αβγ 3
10.   5
11.   HNS 5
12.   10
13.   15
14.   TNF 5
15.   10
16.   15
17.   mw ULTA FREE MC
                30, KD

Put 300λ 1x kinase
buffer in
bottom
    ↓
prevent drying
    filter

Should recover
≤ 40λ retentate
recover
25λ HNS
add 15λ 1x
40λ TNF

Purpose: to compare activity of
yβ vs yβγ vs yαβγ vs HNS vs TNF

10% gel (10-10)		Stack
30% acryl	5ml	1.05
8.8	3.75	1.9 (6.8)
H_2O	6.25	4.5
APS	100	75
TEMED	10	10

File/Range: D:\Users\1012bsm.gel / 0.000-45853 Counts / 1.000000
User Name: phospho
Image Name: D:\Users\1012bsm.gel
Image Comment: yeast b bg abg HNS TNF-Hela
                  scanned 9:13 am to2:05 pm

Present Date/Time:
Scan Date/Time:
Prep. Date/Time:
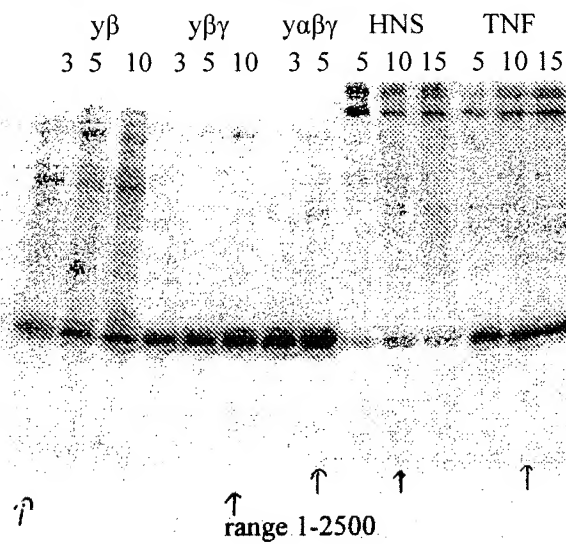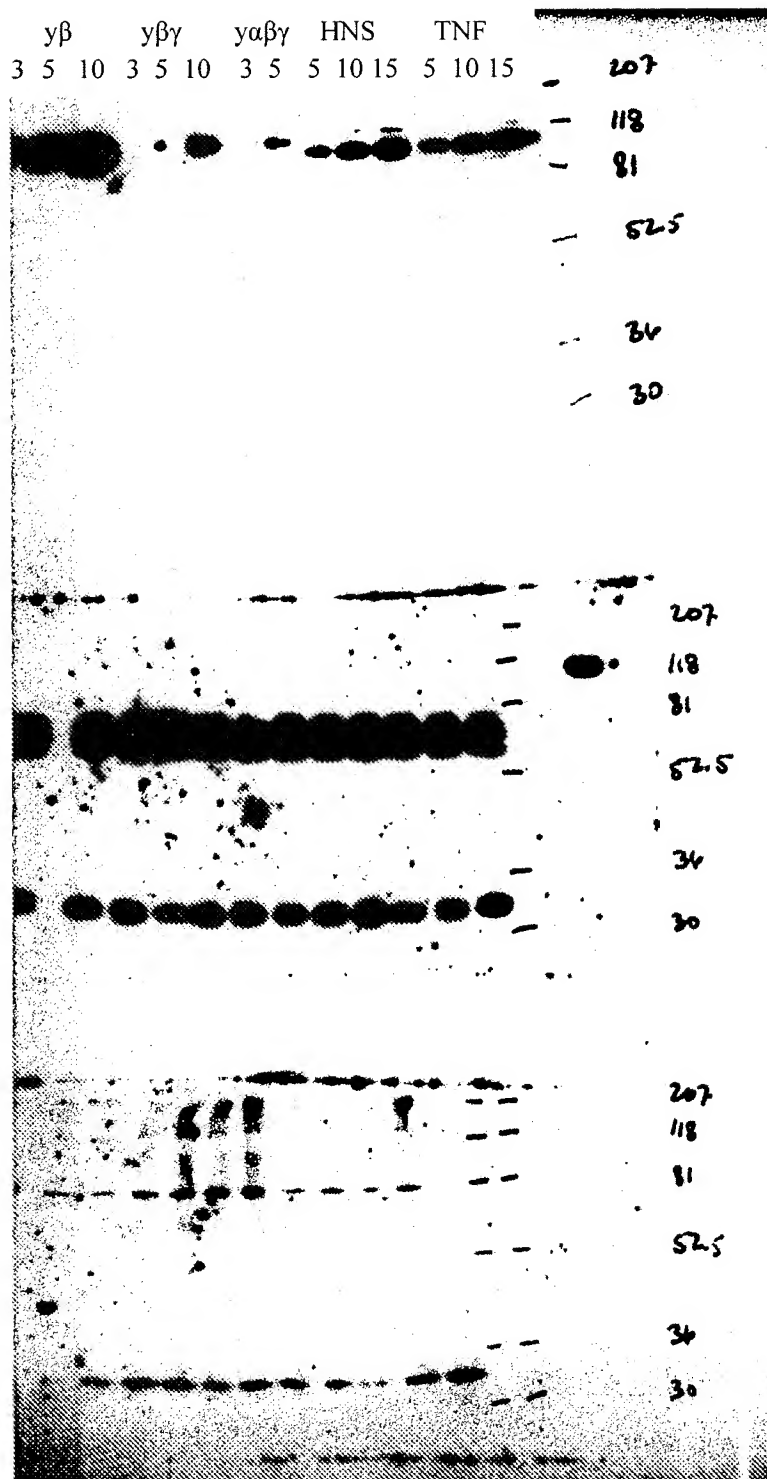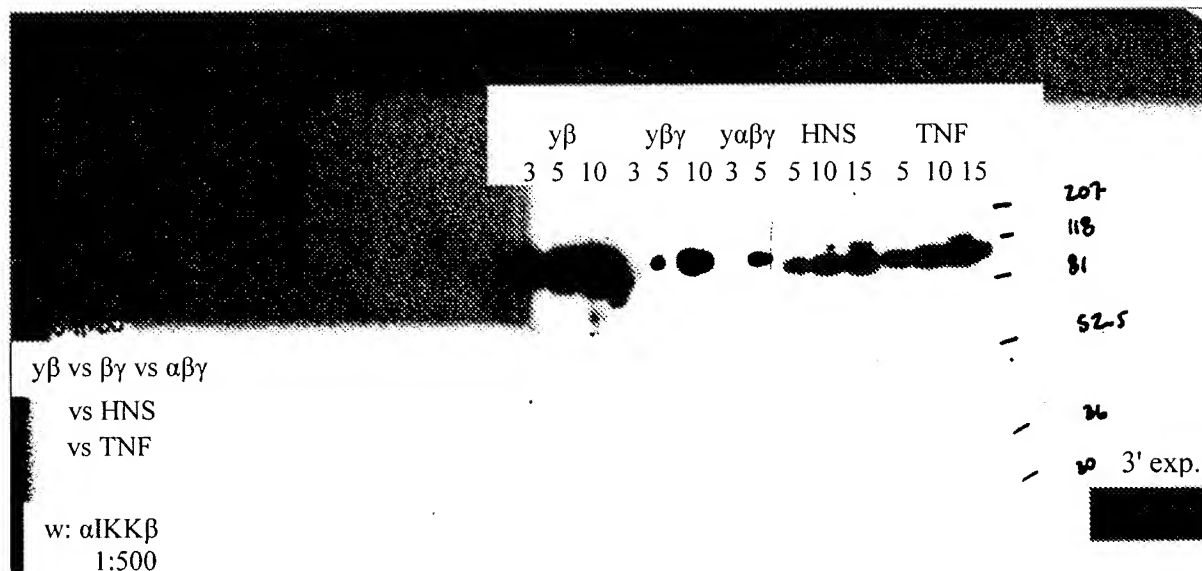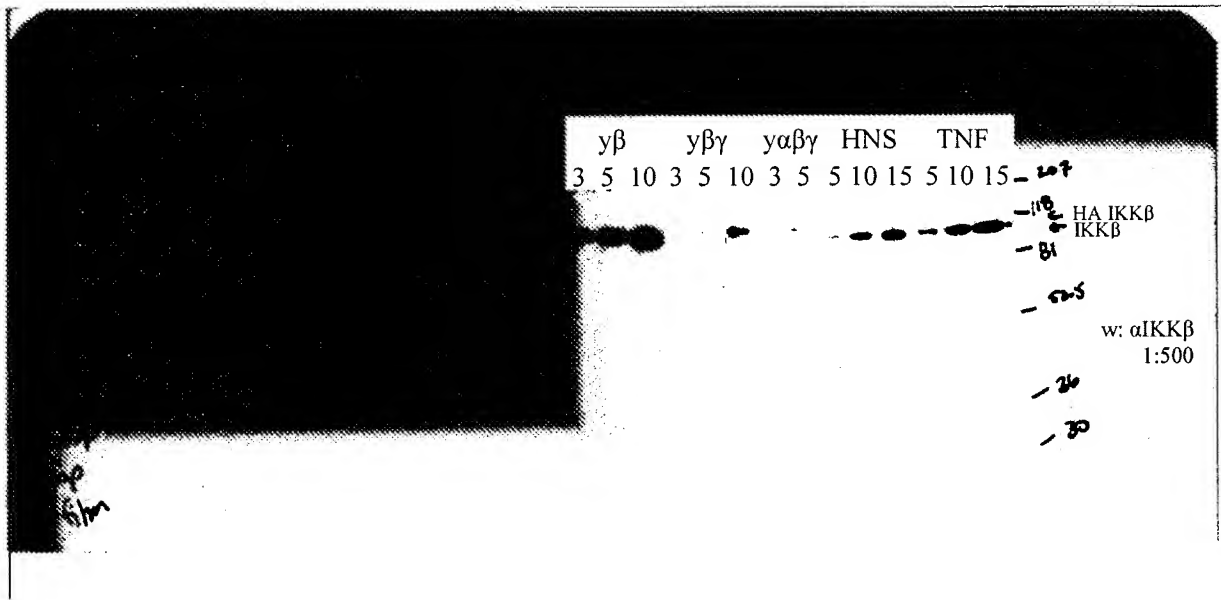

range 1-10,000

File/Range: D:\Users\1012bsm.gel / 0.000-45853 Counts / 1.000000
User Name: phospho
Image Name: D:\Users\1012bsm.gel
Image Comment: yeast b bg abg HNS TNF-Hela
scanned 9:13 am to2:05 pm

Present Date/Time:
Scan Date/Time:
Prep. Date/Time:

range 1-2500

yβ   yβγ   yαβγ HNS   TNF

3 5 10 3 5 10 3 5 5 10 15 5 10 15

207

118

81

52.5

26

10 3' exp.

yβ vs βγ vs αβγ

vs HNS

vs TNF

w: αIKKβ

1:500

Purpose: to compare IKK activity in
yβ vs yβγ vs yαβγ vs HNS vs TNF-Hela

repeat of 10-11 with attempt to use more similar amounts

HNS + TNF (Q20 → sup6 GF 10 + 11)
    Put 300λ 1x 1 kinase buffer in bottom to prevent drying.
    Top: 200λ sup6 GF 10 + 200λ sup6 GF 11

        recover ~ 40λ + adjust vol. to 40λ
                (1x KA)

β − HA fraction 15
        5λ + 45λ 1x
        Dilute 1:10 in 1x kinase

Tube/lane

Tube/lane					
2	0.5λ	5λ + 16λ	1x	1.	Aliquot extract + buffer
3	1λ	10λ + 11λ	1x	2.	Add 35λ kinase
4	2λ	20λ + 1λ	1x		cocktail Jnc 30' 30° C
		equiv. amount		3.	Add 12.2λ 6x
5	βγ − 7λ	~ 5λ (Hela TNF)	+14λ 1x		SDS PAGE
6	14λ	~10	+7λ 1x		Heat 95° C 5'
7	21λ	~15	+0	4.	Load 10% gel
					(40λ)
8	αβγ 7 + 14λ 1x				
9	14 + 7λ 1x				
10	21 + 0				

Cocktail	16 sample	+ 4
10χ Kinase	48λ	12λ
20mm DTT	48λ	12λ
200μm ATP	48λ	12λ
GST − IKβα	32λ	8λ
^{32}P ATP	8λ 906-58	2λ
H₂O	<u>296</u>	<u>74</u>
	480	120

11   HNS 5 + 16λ 1x
12   10 + 11λ 1x
13   15 + 6λ 1x

14   TNF 5 + 16λ 1x
15   10 + 11λ 1x
16   15 + 6λ 1x

20mm DTT
.02 ml 1M + .98ml H₂O

17   MW

   21   all loaded correctly! 40λ each
   <u>35</u>
   56

File/Range: D:\Users\1017bsm.gel / 0.000-45853 Counts / 0.814331
User Name: phospho
Image Name: D:\Users\1017bsm.gel
Image Comment: 2 experiments
   1. 3 M urea GF column fractions (concentrated)
   2. yeast b, b, ab, HNS, TNF stim Hela

Present Date/Time:
Scan Date/Time:
Prep. Date/Time:

TNF-
γβ   γβγ   γαβγ   HNS   HeLa
0.5 1 2 7 14 21 7 14 21 5 10 15 5 10 15

scale 1-2500

β   , βγ   αβγ   HNS   TNF

1λ γαβγ

File/Range: D:\Users\1017bsm.gel / 0.000-45853 Counts / 0.814331
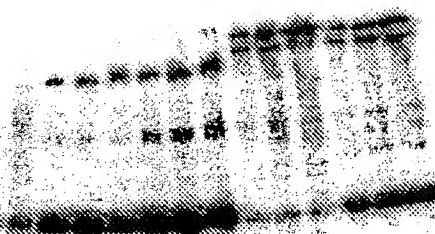User Name: phospho
Image Name: D:\Users\1017bsm.gel
Image Comment: 2 experiments
    1. 3 M urea GF column fractions (concentrated)
    2. yeast b, bg, abg, HNS, TNF stim Hela

Present Date/Time:
Scan Date/Time:
Prep. Date/Time:

scale 1-250

Straight
KA,

w: IKKβ
1:500
O/N

1' exp.